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Effects of linker insertions on the biogenesis and functioning of the *Escherichia coli* outer membrane pore protein PhoE

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Summary. To study the effects of small insertions on the biogenesis and functioning of outer membrane pore protein PhoE of *Escherichia coli* K12, oligonucleotides were inserted at five different sites in the *phoE* gene. The proteins encoded by the mutant alleles all appeared to be incorporated into the outer membrane since cells producing these proteins bound either phages specific for the PhoE protein or monoclonal antibodies or both. However, one mutant protein was apparently not incorporated normally since expression of this protein was lethal and the protein did not function as a pore. Amino acid residues 75 and 280 of the PhoE protein appear to be exposed on the cell surface because insertions at these sites interfere with the binding of PhoE-specific phages or monoclonal antibodies to whole cells, respectively.

Key words: PhoE porin – *Escherichia coli* – Insertion mutagenesis – Topology – Biogenesis

Introduction

The outer membrane of *Escherichia coli* K12 contains a number of proteins which form pores through which small hydrophilic solutes can pass (Nikaido and Vaara 1985). Under standard laboratory conditions, *E. coli* K12 produces the OmpC and OmpF pore proteins. When cells are grown under phosphate limitation another pore protein, PhoE (Overbeeke and Lugtenberg 1980; Tommassen and Lugtenberg 1980), is synthesized which forms particularly efficient channels for negatively charged solutes (Benz et al. 1984; Korteland et al. 1984). Our aim is to identify those domains in the PhoE protein which are important for the biogenesis and for particular functions of the protein.

Like other exported proteins, the PhoE protein is synthesized in a precursor form with an N-terminal signal sequence. Such signal sequences are essential for transport through the cytoplasmic membrane, since mutations in a signal sequence often interfere with this (Bedouelle et al. 1980; Emr et al. 1980). In addition, the signal sequence appears to contain all the information which is essential for export since polypeptides encoded by a set of mutant alleles carrying overlapping internal deletions in the *phoE* gene were all exported from the cytoplasm (Bosch et al. 1986). The observation that these polypeptides all accumulated in the periplasm (Bosch et al. 1986), suggested that the deletions interfere with the normal folding of the protein and thereby prevent its incorporation into the outer membrane.

In this paper, we describe more subtle modifications of the PhoE protein. We created small insertions in the *phoE* gene by means of linker insertion mutagenesis and the products of the mutant alleles were characterized.

Materials and methods

Bacterial strains, plasmids, phages and growth conditions. The *E. coli* K12 strains CE1224 (Tommassen et al. 1983) and CE1248 (Van der Ley et al. 1985) are deleted for the *phoE* gene and do not produce the OmpF and OmpC proteins as a result of *ompR* mutations. CE1248 also carries a *phoR* mutation resulting in constitutive expression of the *pho* regulon. Plasmid pJP29 (Bosch et al. 1986) carries the *phoE* gene and is depicted in Fig. 1. The PhoE-specific phage TC45 (Chai and Foulds 1978) and its host range derivative TC45*hr*N3 were from our laboratory stocks. Phage TC45*hr*N3 recognizes another part of the PhoE protein from TC45 since a substitution of arginine for histidine at amino acid residue 158 renders cells resistant to phage TC45 but sensitive to TC45*hr*N3 and since TC45*hr*N3 recognizes, in contrast to TC45, an OmpF – PhoE hybrid protein (Tommassen et al. 1984). Unless otherwise noted bacteria were grown under aeration at 37° C in L broth (Tommassen et al. 1983) or in a medium described by Levinthal et al. (1962), in which the phosphate concentration is limiting for growth. Where necessary the medium was supplemented with chloramphenicol (25 μg/ml) or ampicillin (50 μg/ml).

DNA techniques. Plasmid DNA was prepared as described by Birnboim and Doly 1979, followed by CsCl-ethidium bromide isopycnic centrifugation.

To create small insertions into the *phoE* gene, 1 μg of pJP29 DNA was linearized with restriction enzymes and protruding ends were either filled in by Klenow fragment of *E. coli* DNA polymerase I or digested using the 3′–5′ exonuclease activity of T4 DNA polymerase, in the presence of 2 mM dNTP. The blunt-ended DNA was ligated with T4 DNA ligase to 100 ng of one of the phosphorylated oligonucleotides dCGGATCCG (*Bam*HI linker), dCGGGATCCCG (*Bam*HI linker) or dCCAAGCTTGG (*Hin*dIII linker). After digestion with *Bam*HI or *Hin*dIII

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and religation with T4 DNA ligase the DNA preparation was used to transform strain CE1224, selecting for chloramphenicol-resistant colonies.

DNA sequencing was performed as described (Sanger et al. 1977).

Isolation and characterization of cell fractions. Cell envelopes were isolated by ultrasonic disintegration of cells followed by centrifugation (Lugtenberg et al. 1975). A fraction containing outer membrane proteins was isolated by Triton X-100 extraction of cell envelopes (Schnaitman 1974). The protein patterns of the cell fractions were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Lugtenberg et al. 1975).

Pulse-label and pulse-chase experiments. Pulse-label and pulse-chase experiments were performed as described (Bosch et al. 1986).

Phage sensitivity and adsorption. The sensitivity of strains to phages was determined by cross-streaking on plates. Irreversible phage binding to cells carrying the insertion plasmids was measured by incubating the cells with a phage suspension and subsequent titration of the remaining phages (Van der Ley et al. 1985).

Immunological assays. The cell immunoradio assay (CIRA) was performed as described (Van der Ley et al. 1985), except that cells were grown in phosphate-limited medium.

Uptake of β-lactam antibiotics. The rate of permeation of the β-lactam antibiotics cefsulodin and cephaloridine through the mutant pores was determined in derivatives of strain CE1248 carrying pJP29 with the different *phoE* alleles. The rate of uptake can be determined by measuring the decolourisation of an indicator solution due to the degradation of the β-lactam antibiotics in the periplasm by the periplasmic β-lactamase provided by plasmid pBR322. The method was originally described by Zimmerman and Rosselet (1977) and modified by Overbeeke and Lugtenberg (1982).

Results

Construction of phoE insertion mutations

To create small amino acid insertions into the PhoE protein, oligonucleotides were introduced at the five unique restriction sites in the *phoE* gene carried on plasmid pJP29 (Bosch et al. 1986; Fig. 1). The plasmid was linearized with one of these enzymes, the protruding ends were filled in or digested and a DNA linker was inserted as described in Materials and methods. To maintain the correct reading frame, either 8 or 10 bp oligonucleotides were used. All constructions were verified by DNA sequence analysis. In all cases, the mutagenesis resulted in the addition of four amino acids into the PhoE protein, except for the insertion at the *Pst*I site, which resulted in the replacement of one amino acid by three other residues (Table 1).

Expression of the mutant gene products

To identify the polypeptides encoded by the mutant alleles, cell envelope proteins were analysed from CE1224 cells carrying the insertion plasmids after growth under phosphate limitation (Fig. 2). As compared with the wild-type PhoE protein (lane C), the mutant proteins showed an identical or a lower electrophoretic mobility (lanes D to I). The possibility that these slower migrating proteins represented unprocessed precursors of the mutant proteins was ruled out by performing pulse-label and pulse-chase experiments

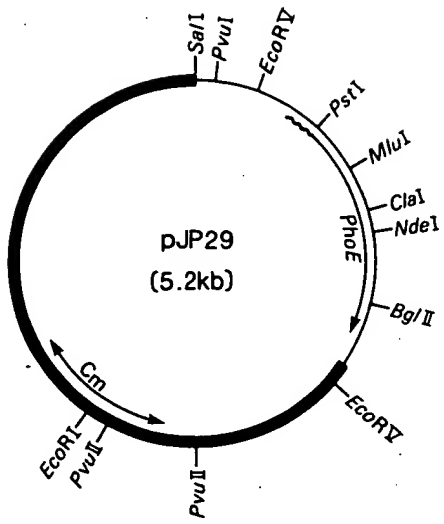

Fig. 1. Physical map of pJP29 (Bosch et al. 1986). The *thick line* represents DNA derived from the cloning vector pACYC184; the *thin line* represents chromosomal DNA. The position and direction of transcription of *phoE* are indicated by an *arrow*. The *wavy line* represents DNA coding for the signal sequence. The position of the chloramphenicol resistance marker (Cm) is also indicated

Table 1. Derivatives of plasmid pJP29 carrying insertions in the *phoE* gene

Site of mutagenesis[a]		Mutant plasmid designation	Structure of the insertion[b]			
DNA level	Protein level					
*Pst*I	1	pJP29-P1	GGG Gly	ATC Ile	CCG Pro	
*Mlu*I	74–75	pJP29-M1	CGC Arg	GGA GLy	TCC Ser	GCG Ala
*Cal*I	142–143	pJP29-C1	GCG Ala	GGA Gly	TCC Ser	CGC Arg
*Cla*I	142–143	pJP29-C2	GCC Ala	AAG Lys	CTT Leu	GGC Gly
*Nde*I	173–174	pJP29-N1	TAC Tyr	GGG Gly	ATC Ile	CCG Pro
*Bgl*II	279–280	pJP29-B1	GAT Asp	CCG Pro	GAT Asp	CCG Pro

[a] For the DNA level, the restriction site in plasmid pJP29 (Fig. 1) which was used for insertion mutagenesis is indicated. The numbers of the corresponding amino acid residues in the mature PhoE protein are indicated for the protein level. The wild-type mature PhoE protein consists of 330 amino acid residues (Overbeeke et al. 1983).

[b] Bases and amino acids inserted in the *phoE* gene and PhoE protein, respectively. In the pJP29-P1-encoded mutant protein, the first amino acid residue of the wild-type mature protein, an alanine residue, is substituted by glycine, isoleucine and proline residues

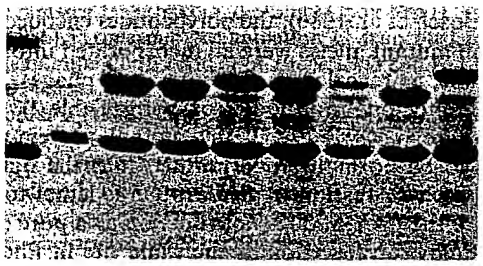

Fig. 2. SDS-polyacrylamide gel electrophoresis patterns of cell envelope proteins of plasmid-containing derivatives of strain CE1224 after growth under phosphate limitation. Plasmids present were pACYC184 (lane B), pJP29 (C) and the mutant plasmids pJP29-P1 (D), pJP29-M1 (E), pJP29-C1 (F), pJP29-C2 (G), pJP29-N1 (H) and pJP29-B1 (I). Lane A conains the molecular weight standard. The molecular weights of the standard proteins as well as the position of the wild-type PhoE protein are indicated on the left

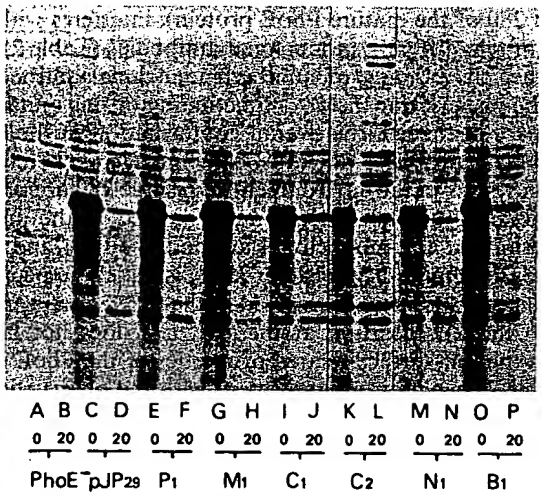

Fig. 3. Autoradiogram of ^{35}S-labelled proteins from whole cells of strain CE1224 separated by SDS-polyacrylamide gel electrophoresis. The cells contained plasmid pACYC184 (lanes A and B), pJP29 (lanes C and D) or the indicated mutant derivatives of pJP29: pJP29-P1, pJP29-M1, pJP29-C1, pJP29-C2, pJP29-N1 and pJP29-B1. The cells were starved for phosphate and pulse labelled with [^{35}S]-methionine for 30 s. The pulse was followed by chase periods of 0 or 20 min as indicated

(Fig. 3). When cells containing pJP29 were pulse labelled for 30 s with ^{35}S-methionine under conditions of phosphate starvation and total cellular proteins were separated on SDS-polyacrylamide gels, two bands, representing the precursor form and the mature form of PhoE protein, were detected on the autoradiogram of the gels (lane C). The precursor disappeared during a 20 min chase period (lane D). Similarly, a transient precursor form could be detected for all mutant proteins (lanes E to P).

Except in the case of pJP29-C2, all mutant proteins were constitutively expressed in *phoR* mutant strain CE1248 (not shown). It was impossible to transform the latter strain with pJP29-C2. Apparently, expression of the corresponding mutant PhoE protein is lethal to the cells. This probably explains the observed decreased amount of mutant protein in pJP29-C2-carrying cells (Fig. 2, lane G).

Table 2. Interaction of cells carrying the mutant plasmids with phages and monoclonal antibodies

Plasmid	Phage receptor activity[a]		Monoclonal antibody binding[b]		
	Percentage of phages bound in adsorption test	Sensitivity to phages	PP1-4	PP2-1	PP3-4
pACYC184	< 5	R	4	7	5
pJP29	>98	S	177	200	195
pJP29-P1	>98	S	185	215	195
pJP29-M1	< 5	R	182	213	205
pJP29-C1	>98	S	196	212	221
pJP29-C2	< 5	–	93	33	72
pJP29-N1	>98	S	182	207	187
pJP29-B1	>98	S	6	217	3

[a] The interaction of the mutant proteins to PhoE-specific phages TC45 and TC45*hr*N3 was tested. There was no detectable difference in interaction between these two phages and the mutant cells. The phage adsorption was measured with phosphate-limited CE1224 cells carrying the mutant plasmids. Sensitivity to phages was tested with derivatives of strain CE1248, except for the pJP29-C2 mutant plasmid which could not be introduced in to this strain. S, sensitive; R, resistant

[b] Monoclonal antibody binding was measured in CIRA experiments. Cells were incubated with diluted ascites fluid and subsequently with ^{125}I-labelled protein A. The amount of radioactivity bound to the cells after washing is given $\times 10^{-3}$ cpm

Subcellular localization and characterization of the mutant PhoE proteins

From the pulse-label experiments (Fig. 3), it appears that all mutant proteins are normally processed, suggesting transport through the cytoplasmic membrane. Cell fractionation experiments localized the mutant protein in the outer membrane, since the proteins were present in the Triton X-100 insoluble fraction of the cell envelopes (not shown). However, since localization of mutant proteins by cell fractionation experiments may be unreliable (Bosch et al. 1986; Tommassen et al. 1985), functional assays are required to confirm the presence of these proteins in the outer membrane.

The PhoE protein serves as (part of) the receptor for phage TC45 and its host range derivative TC45*hr*N3. Phage adsorption experiments were performed with phosphate-limited CE1224 cells carrying the mutant plasmids (Table 2). The phages bound to cells carrying pJP29, pJP29-P1, pJP29-C1, pJP29-N1 and pJP29-B1 but not to cells carrying pJP29-M1 and pJP29-C2. Consistent with these results, derivatives of the *phoR* mutant strain CE1248 carrying any one of the five former plasmids were sensitive to the phages, in contrast to the derivative carrying pJP29-M1 (Table 2).

The binding of three different monoclonal antibodies, PP1-4, PP2-1 and PP3-4, which recognize the part of the PhoE protein exposed at the cell surface (Van der Ley et al. 1985), to phosphate-limited CE1224 cells carrying the insertion plasmids, was studied in CIRA experiments (Table 2). Only the binding of antibodies to cells carrying pJP29-C2 and pJP29-B1 appeared to be disturbed. The pJP29-C2-carrying strain showed reduced binding of all the anti-

Table 3. Rate of permeation of β-lactam antibiotics through the mutant pores in intact cells

Plasmid	Rate of uptake in intact cells[a]		Pore specificity determined by Vu/Va
	Cefsulodin (Vu)	Cephaloridine (Va)	
pACYC184	0.6	1.8	–
pJP29	11.2	6.2	1.8
pJP29-P1	11.5	6.2	1.8
pJP29-M1	5.5	9.7	0.6
pJP29-C1	5.6	5.8	1.0
pJP29-C2[b]	0.6	1.5	–
pJP29-N1	11.5	6.1	1.9
pJP29-B1	11.7	6.3	1.8

[a] Rate of permeation of the β-lactams is expressed in nmol per min per 10^8 cells

[b] Pore characteristics of pJP29-C2-encoded mutant proteins were measured in CE1224 cells grown on medium described by Levinthal et al. (1962). No difference in β-lactam permeation was observed between the wild-type PhoE-producing CE1224 cells grown on this medium and the wild-type PhoE-producing *phoR* strain CE1248 grown on L broth

bodies, probably because of the relatively low amount of mutant PhoE protein produced by this strain (Fig. 2, lane G). The pJP29-B1-containing cells bound only monoclonal antibody PP2-1. Apparently, the insertion in this plasmid disturbs the antigenic determinant recognized by the antibodies PP1-4 and PP3-4. Since in all cases, at least one monoclonal antibody bound to whole cells producing the altered PhoE proteins, it appears that all these proteins are incorporated into the outer membrane.

Pore function of the altered PhoE proteins

The PhoE protein forms pores in the outer membrane with a preference for anionic solutes (Benz et al. 1984; Korteland et al. 1984). The specificity and the efficiency of the pores can be demonstrated by measuring the rates of permeation of the β-lactam antibiotics cephaloridine and cefsulodin in cells producing only one porin species. Cefsulodin is closely related to cephaloridine but contains an additional negative charge and its molecular weight is higher (Nikaido et al. 1983; Overbeeke and Lugtenberg 1982). The rate of uptake of the β-lactams by pJP29-P1-, pJP29-N1- and pJP29-B1-carrying cells did not significantly deviate from that of the strain producing wild-type PhoE protein (Table 3). However, the rate of uptake of cefsulodin by pJP29-C1- and pJP29-M1-carrying cells was significantly decreased and cells carrying the latter plasmid show an increased uptake of cephaloridine. The specificity of the pores encoded by the latter two plasmids, expressed by the relative rates of uptake of the two antibiotics, also appears to be changed. No pore activity could be detected in pJP29-C2-carrying cells (Table 3).

Discussion

To study the export process, the topology and the structure-function relationship of the outer membrane protein PhoE, a number of linker insertion mutations into the *phoE* gene were created. In contrast to the products of mutant alleles with internal *phoE* gene deletions, described in a previous study (Bosch et al. 1986), the polypeptides encoded by these insertion mutant alleles were all incorporated into the outer membrane, as revealed by the binding of phages, the binding of monoclonal antibodies or both (Table 2). However in one case, i.e. pJP29-C2, the mutant protein does not appear to be normally incorporated into the outer membrane, since expression of this protein is lethal to the cells and since the protein does not function as a pore (Table 3). Interestingly, an insertion at the same site in PhoE of approximately the same amino acids but in a different order, i.e. in plasmid pJP29-C1 (Table 1), does not interfere with the normal incorporation of the protein. Although the molecular mechanism of this interference is not understood at the moment, the lethality of expression of the mutant protein offers an opportunity to select for revertants which do incorporate the protein normally into the outer membrane. We expect that analysis of such revertants will lead to a deeper understanding of the structural requirements for incorporation of the PhoE protein into the outer membrane.

The insertion at the *BglII* site (corresponding to amino acid 280 of the mature PhoE protein), interferes with binding of two different monoclonal antibodies (Table 2). Since neither the binding of another monoclonal antibody and of the phages, nor the pore functioning of this mutant protein are disturbed, the overall conformation of the mutant protein is probably not drastically changed. Therefore the amino acids in the environment of residue 280 are probably exposed at the cell surface and involved in the antigenic determinant for monoclonal antibodies PP1-4 and PP3-4. Similarly, amino acid residue 75 is also likely to be exposed at the cell surface and involved in the phage receptor activity (Table 2). Insertion at the *PstI* site does not interfere with the cell-surface related properties of the PhoE protein (Table 2), which is an agreement with the observation that the N-terminus of the protein is probably exposed on the periplasmic side of the membrane (Tommassen and Lugtenberg 1984).

The pore characteristics of the mutant proteins were slightly modified in two cases (Table 3). The decreased rate of uptake of cefsulodin by cells carrying pJP29-C1 may be explained by a narrowing of the channel by the addition of four amino acids. The simultaneous decrease in cefsulodin uptake and the increase in cephaloridine uptake by pJP29-M1 is more difficult to understand. Shielding of amino acids involved in the anion specificity of the PhoE pores might be an explanation.

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